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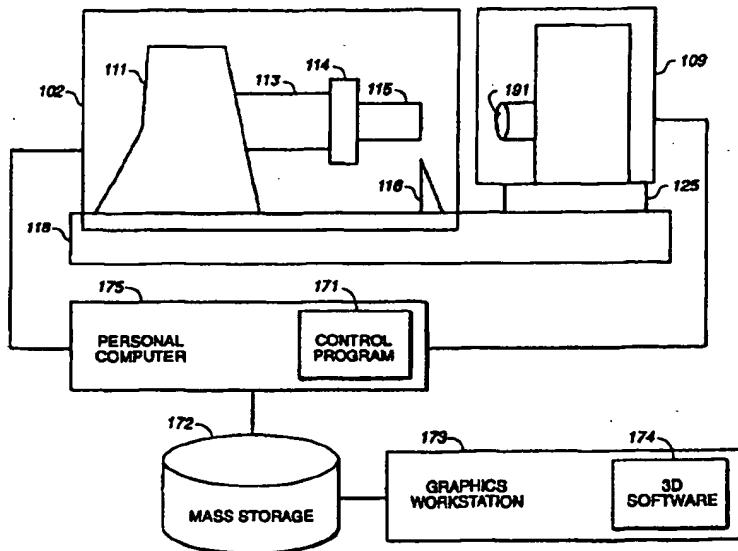


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(54) Title: IMAGE RECORDING WITH OPTICAL SECTIONING



(57) Abstract

A method and apparatus for imaging an object with a planar sectioning microscope (109). The method locates a plane of best focus of the microscope at sequential planes within the object and images the object at each plane. A slice is removed from the object with a microtome (102), the slice including a section corresponding to at least one of the previously imaged planes. The plane of best focus is translated to a third plane within the object, and another plane is imaged. The apparatus includes a microtome (102) for cutting a slice from the object upon command of a control program, a planar sectioning microscope (109) having a plane of best focus and a narrow depth of focus for imaging the object, and an optical mount for adjustably translating the plane of best focus relative to the object (125).

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## IMAGE RECORDING WITH OPTICAL SECTIONING

DESCRIPTION5 TECHNICAL FIELD

The present invention relates to optical microscopes for imaging sectioned objects.

BACKGROUND ART

10 The microscope is a historic and powerful tool for analyzing small objects. Researchers such as biologists and pathologists typically use microscopes to examine features of biological tissues. Commonly, thinly cut tissue samples are viewed as prepared on a microscope slide. These slides present a cross-sectional view of the tissue sample from a single angle defined by the angle at 15 which the sample was cut.

Optical microscopes use reflected or transmitted light to obtain structural information about the sample. 20 Light rays can penetrate only a limited distance into a tissue sample. the actual limit is highly dependent on the type of tissue being examined, but a general limit of approximately 0.5 mm is typical.

Other factors also affect depth resolution of 25 microscopes. For example, the often small working distances of high-powered microscope lenses can physically limit the depth to which the lenses may be focused because the lens may physically contact the sample. Further, most microscopes have a limited depth of focus. The tissue 30 layers overlying and underlying the focused layers can contribute noise to the image and cause overall image degradation.

For a variety of reasons, out-of-focus objects 35 within the overlying or underlying layers can contribute to localized defects or artifacts in the image. These defects and artifacts can cause the recorded image to be an inaccurate representation of the actual object.

Even without such degradations of the image

data, the identification of specific tissue features often greatly depends on the angle at which the researcher views the tissue section. This angle, in turn, depends on the angle at which the tissue section was cut by the technician. It is thus desirable for the technician making the section to have knowledge of which angles are preferable, if any. It is also desirable for the technician to prepare the sample in a careful manner.

This type of care is time and labor intensive. 10 Mistakes, such as cutting a sample at a less desirable angle, often cannot be fixed. An entirely new section may be required.

Some solutions have been suggested to ease the pathologist's dependence on the skill of the technician. 15 For example, U.S. Patent No. 4,960,330 to Kerschmann, which is incorporated by reference, discloses a pathology image recording apparatus. The apparatus stores microscopy data of successive sections of an object for three-dimensional processing. The researcher may 20 arbitrarily rotate and view the image of the object or the image of any plane through the object. This may make the usefulness of the image less dependent on the skill of the technician.

The '330 patent teaches staining the object 25 block with a fluorescent dye, causing the stained block to fluoresce, and imaging the surface of the block as a microtome removes successive sections. Layers below the surface of the block also fluoresce in response to illumination.

30 The '330 patent teaches several techniques to ensure that the surface plane provides the primary contribution to the overall image. In these, the contribution of the surface plane to the image is enhanced and that of the planes below are reduced. First, the 35 microscope is focused at the surface plane, making the surface plane contribution the clearest. Second, the object is prepared so as to reduce the interior layers' contribution. For example, the stained tissue is

surrounded with and embedded in a medium treated with or containing a quencher. The quencher inhibits excitation by the dye to a greater extent in the deep tissue layers than at the surface.

5

#### DISCLOSURE OF INVENTION

In one aspect, the present invention is directed to a method for imaging an object with a planar sectioning microscope. The method comprises the steps of locating a 10 plane of best focus of the microscope at a first plane within the object, taking an image of the object at the first plane, translating the plane of best focus to a second plane within the object, taking an image of the object at the second plane, removing a slice from the 15 object with a microtome, the slice including a section corresponding to at least one of the first or second planes, translating the plane of best focus to a third plane within the object, and taking an image of the object at the third plane.

20

Implementations of the invention include the following features. The method may include the step of preparing the object by staining the object with a dye and infiltrating the object with a transparent medium. The method may also include the step of embedding the object 25 in a transparent medium. The method may further include the step of processing the images detected. The method may include the step of using the images stored to create a model of the object or a plane within the object on a computer screen.

30

In another aspect, the invention is directed to an apparatus for optically and mechanically sectioning an object. The apparatus comprises a microtome having a holder for receiving the object, the microtome for cutting a slice from the object upon command of a control program. 35 The apparatus also comprises a planar sectioning microscope mechanically coupled to the microtome by a bench and having a plane of best focus and a narrow depth of focus for imaging the object, and an optical mount

mechanically coupled to the planar sectioning microscope and the bench for adjustably translating the plane of best focus relative to the object.

Implementations include the following features.

5 The planar sectioning microscope may be a confocal microscope. The confocal microscope may be a laser scanning confocal microscope. The laser scanning confocal microscope may include a photodiode detector or a photomultiplier detector. The apparatus may further  
10 comprise a film mount for holding film on which images from the planar sectioning microscope may be recorded.

In another aspect, the invention is directed to another method for imaging an object. This method comprises the steps of preparing the object, placing the  
15 object in a holder for viewing by a planar sectioning microscope having a plane of best focus and a narrow depth of focus, receiving by the planar sectioning microscope an image of a first plane of the object, the first plane located at a plane of best focus, moving the plane of best  
20 focus to a second plane of the object, receiving via the planar sectioning microscope an image of the second plane of the object, cutting a slice from the object with a microtome, the slice including at least one of the first or second planes, moving the plane of best focus to a  
25 third plane of the object, receiving via the planar sectioning microscope an image of the third plane of the object, and storing the images so received in a computer.

Implementations may include the following features. The method may include the step of staining the  
30 object with a dye. The holder may be made of an optically clear material.

In another aspect, the invention is directed to another method for imaging an object with a planar sectioning microscope. The method comprises the steps of receiving by the planar sectioning microscope an image of a surface plane of the object, translating a plane of best focus to a second plane within the object, receiving by the planar sectioning microscope and image of the object

5 at the second plane, removing a slice from the object with a microtome, translating the plane of best focus to a third plane of the object, and receiving by the planar sectioning microscope an image of the object at the third plane.

In another aspect, the invention is directed to another method for imaging an object with a planar sectioning microscope. This method comprises the steps of receiving by the planar sectioning microscope an image of 10 a first plane of the object, translating the plane of best focus by a first increment to a second plane of the object, receiving by the planar sectioning microscope an image of the object at the second plane, translating the plane of best focus by a second increment to a third plane 15 of the object, receiving by the planar sectioning microscope an image of the object at the third plane, removing a slice from the object with a microtome, the slice including a section corresponding to a least one of the first, second, or third planes, translating the plane 20 of best focus to a fourth plane within the object, and receiving by the planar sectioning microscope an image of the object at the fourth plane.

Among the advantages of the invention are the following. A three-dimensional model of a tissue sample 25 may be made without mechanically slicing the sample to obtain each image plane of data. This may be especially advantageous if the sample is delicate and not easily sliced. High-depth-resolution computer models of the object may be created. The usefulness of the data is less 30 dependent on the skill of the technician preparing the sample.

Additional advantages of the invention will appear from the description which follows. The advantages 35 of the invention may be realized and obtained by means of the instrumentalities and combinations particularly pointed out in the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a side view of a cutting system and microscope according to an embodiment of the invention.

5 Fig. 2 is a flowchart of a control program for an image gathering process according to an embodiment of the invention.

Fig. 3 is a flowchart of an image capture, processing, and storage procedure according to an embodiment of the invention.

10

BEST MODE FOR CARRYING OUT THE INVENTION

Referring to Fig. 1, a system for imaging sectioned objects includes microtome 102 and planar sectioning microscope 109, each mounted on bench 118.

15 Microtome 102 holds a tissue block 115 in a holder 114 generally facing planar sectioning microscope 109. The term "tissue block" refers to a tissue sample prepared for cutting, typically combined with a hardening material, such as an infiltrating/embedding medium. The optical 20 axis of planar sectioning microscope 109 generally intersects tissue block 115 and for convenience is identified with the z-axis.

A planar sectioning microscope is one that has a narrow depth of focus, often half that of a conventional 25 microscope. The in-focus volume seen by a planar sectioning microscope is wide in two dimensions but narrow in a third, roughly forming a plane. If the depth of focus is sufficiently narrow, the planar sectioning microscope can be used to selectively examine a thin plane 30 in an object, referred to here as the "plane of best focus". The thickness of this plane, that corresponds to the narrow depth of focus, may be, e.g., half a micron. The image of the plane of best focus is received in planar sectioning microscope 109 through lens 191. Planar 35 sectioning microscope 109 may be, for example, a laser scanning confocal microscope (LSCM) such as those manufactured by Zeiss, Biorad, or Olympus. Other types of planar sectioning microscopes can be used, including

optical sectioning microscopes.

The position of the plane of best focus may be moved to different points within the object by several techniques. For example, the LSCM can be mounted on an 5 optical mount, allowing the LSCM to be moved relative to the object. Moving the LSCM accordingly moves the position of the plane of best focus a corresponding amount. In this situation, the distance between the LSCM and the object changes while the distance between the LSCM 10 and the position of the plane of best focus remains constant. In another technique, the plane of best focus may also be moved to different points within the object by adjusting the optics within the LSCM. In this situation, the distance between the LSCM and the object remains 15 constant while the distance between the LSCM and the position of the plane of best focus changes. In any case, the smallest distance that the plane of best focus may be translated is often about a tenth of a micron.

The images produced by planar sectioning 20 microscope 109 may be captured for later playback. In the embodiment that will be described, an LSCM has a photodiode or photomultiplier tube whose digital output is transmitted to a personal computer 175 and stored on a mass storage device 172. However, the microscope output 25 may also be detected by, for example, CCD detectors, especially where an optical sectioning microscope is used.

The detected image may be processed by an image 30 processing device (not shown) prior to transmission to computer 175. In addition, images may be recorded in an intermediate form, such as photographic film, and only later digitalized for storage and processing in a digital format.

A microtome 102 is a cutting device having an 35 automatic advancing mechanism and a cutting blade. Microtomes which may be used include, for example, LEITZ Models 1512 and 1516, as well as those manufactured by Olympus. The slicing thickness of typical microtomes is commonly on the order of a micron, although

ultramicrotomes can be used with slicing thicknesses on the order of tens of nanometers.

Components of microtome 102 include the conventional holder 114, reciprocating bar 113, motor drive 111, and microtome blade 116. Motor drive 111 and blade 116 are each mounted to bench 118. An optical mount 125 is also mounted to bench 118 and is coupled to planar sectioning microscope 109 for translating the plane of best focus relative to tissue block 115. Optical mount 125 may translate the plane of best focus in two ways. First, optical mount 125 could include a stepper motor which rotates the focusing knob on the microscope. Second, optical mount 125 could include a stepper motor which moves the microscope forward or back on a translation table. Alternatively to these two ways, a mount could be provided to move microtome 102 instead of, or in conjunction with, planar sectioning microscope 109. Nevertheless, consistently moving tissue block 115 may result in degradation of delicate samples. Thus, the first techniques may be more convenient.

The object to be imaged can be prepared to enhance its viewability. Such preparation normally includes staining and infiltration/embedding. Standard techniques can accomplish these tasks.

Normally a tissue sample is stained with a dye so as to improve the contrast of the tissue against a background. Staining improves resolution and contrast by introducing differences in optical density or in light absorption between the tissue and the background. It can also provide contrast between the tissue sample and the hardening medium.

The stains used can be brightfield dyes such as those conventionally used. These dyes absorb and emit light at the same wavelength. Therefore, light emitted by brightfield dyes at the plane of best focus may be partially re-absorbed by surrounding layers.

Therefore, better quality images will usually be produced by using fluorescent dyes which absorb and emit

at different wavelengths. This property, the Stokes' shift, reduces the re-absorbance of the emitted light by surrounding tissues. Fluorescent dyes which may be used include acridine orange, rhodamine, eosin, or ethidium bromide.

5 A staining bath may be used to stain the tissue sample and may have only a single stain or may be composed of a number of different stains. These different stains may have different chemical and fluorescence properties 10 selected to differentially stain the tissue structures of interest. If stains are used whose image colors are less familiar to pathologists, computer-implemented image processing techniques can filter and transform the raw 15 image into an image that has more familiar stain colors. This computational manipulation is described below.

The sample may also be infiltrated and embedded. A tissue sample is infiltrated by a material to strengthen and harden the tissue sample. Often, the material is designed to permeate the entire sample. Absent 20 infiltration, a tissue sample may tend to collapse upon itself.

Similarly, embedding places a tissue sample in a material so as to provide protection and strength to the tissue sample. Absent embedding is often used to provide 25 a convenient frame for a tissue sample prior to placement in a microtome holder.

The embedding material can be a polymeric plastic material such as the glycomethacrylates or paraffin used in pathology tissue embedding for light 30 microscopy. Both the embedding material and also the holder 114 can be made of an optically clear material. The infiltrating material may be the same as the embedding material.

A number of techniques can be used to illuminate 35 a sample. Preferably, however, fluorescence is used. In this mode, illumination such as ultraviolet or short-wavelength blue light from a laser excites the fluorescent dye in the tissue. Only the light emitted by the excited

dye is imaged and recorded.

Tissue block 115 is placed in holder 114 in a desired orientation, although a specific orientation is not required. A user may provide a rough alignment of tissue block 115 with planar sectioning microscope 109. Often this alignment involves moving planar sectioning microscope 109 so that the plane of best focus is at the surface of tissue block 115 or within the interior as the user desires.

The operation of the system can be controlled by a control program 171 running on a computer 175. For example, a personal computer 173 such as a PowerMac can be used to coordinate the components and capture the image data. Control program 171 can also run on a graphics workstation 173 later used for viewing, such as a Silicon Graphics' Indigo II High-Impact workstation. The tasks involved in coordinating the components include causing the movement of optical mount 125. Optical mount 125 in turn moves the plane of best focus to a different point within tissue block 115 by, for example, altering the optics within microscope 109. Actions which may also be involved are causing the movement of tissue block 115 and initiating microtome blade slices.

Referring to Fig. 2, control program 171 obtains the operating parameters (step 214) by, for example, querying the user or a data file. These parameters include the depths at which images are taken and the depths at which microtome slices are made. For example, in a sample region of interest, the user may choose to separate images by small increments to obtain the highest resolution.

Once the operating parameters are imputed, control program 171 is started. Control program 171 may initiate the mechanical movement of microtome 102 or it may be initiated manually, i.e. by the user operating microtome 102. Motor drive 111 drives reciprocating bar 113 up and down so that blade 116 cut slices from tissue block 115. After each cut, tissue block 115 is normally

5 advanced a pre-specified distance by an automatic advancing mechanism. The distance advanced is often equal to the preset slicing thickness of microtome 102. In this case, the surface of tissue block 115 is at the same location after each slice is cut.

10 A plane of the object is imaged (step 216) and can be displayed in more than one place. A rough representation of lesser quality may be shown on a local display monitor as a check on the operation of the equipment (step 230). Another image, containing the full recorded digital information, can be sent to computer 175 to be captured (step 232). The image capture software may be, for example, that produced by Leaf Systems, a division 15 of Sci-Tex Inc., for use with their cameras.

15 Once the image of a plane has been captured, control program 171 determines if the task is finished (step 222). The program may terminate if no more images are to be captured (step 228). If more images are to be captured, control program 171 may initiate certain 20 actions. The choice depends on whether the operating parameters cause tissue block 115 to be sliced (step 224). For example, control program 171 may cause a microtome slice of tissue block 115 (step 235).

25 A microtome slice may be desired for two reasons. A first reason is so that planar sectioning microscope 109 can be moved to tissue block 115 without contacting the block. In many planar sectioning microscopes, the distance between the object and the microscope lens is very short. The removal of a surface 30 layer allows the lenses of planar sectioning microscope 109 to be moved sequentially closer to the block.

35 A second reason for a microtome slice is to remove layers of tissue that may absorb light from and degrade the image of deep interior layers. For example, in the examination of deep layers light may be absorbed or scattered by the tissue block volume between the plane of best focus and the surface. This absorption or scattering is more pronounced than in the situation where planar

sectioning microscope 109 is only examining the surface or shallow layers of tissue block 115. The sequential removal of portions of tissue block 115 minimizes the effect of this absorption or scattering.

5 Once the imaging of a portion of tissue block 115 has been completed, that portion may be removed and discarded or saved for future study as necessary. The use of a microtome is especially useful in this connection as microtome slices are particularly well-suited to the 10 manufacture of microscope specimens. The precision of most modern microtomes further allows slices to be made without inducing roughening in the remaining surface of the tissue block.

15 After a slice, planar sectioning microscope 109 is reset (step 225) such that the plane of best focus is again at or within tissue block 115. Another image may then be taken (step 216). If no microtome slice occurs, the plane of best focus is moved to a different depth within tissue block 115 (step 220) and another image taken 20 (step 216).

Each image plane is detected and captured by the computer 175 (step 232). The image data is supplemented with information regarding the depth where the image was captured.

25 The image can be further processed just after the image has been captured, or after the image has been stored. In the latter case, the image data stored in mass storage 172 may be recalled by graphics workstation 173 for further processing and presentation to the user.  
30 Referring to Fig. 3, color processing of images can be performed to enhance features and make the resultant displayed representation have colors similar to the dyes used in conventional pathology microscopy (step 234). Such use of familiar colors yields a comfortable and 35 familiar representation, making use of the device considerably easier.

Resolution of certain features (for example, cell features recognizable by their geometry) may be

enhanced using image enhancement techniques (step 236). Image enhancement techniques may also be used to remove scattered light from out-of-focus tissues lying above or below the layer currently undergoing enhancement.

5 The image data may be stored as received or, optionally, compressed (step 238). Such compression can be substantial due to the large amount of redundancy in such three-dimensional image data. For example, the JPEG technique may be used to compress data of a single image  
10 plane. The MPEG technique may be used to compress data of multiple planes or of a reconstructed three dimensional model of the object.

After storage of the processed data (step 240), the array of sections can be retrieved for playback as a  
15 three-dimensional image on graphics workstation 173 (step 242) using three-dimensional representations of the tissue sample.

20 The present invention has been described in terms of a preferred embodiment. The invention, however, is not limited to the embodiment depicted and described. For example, various other types of microscopes have a narrow depth of field that can image interior sections. Also, numerous types of light detectors may be employed.

## IMAGE RECORDING WITH OPTICAL SECTIONING

CLAIMS

What is claimed as invention is:

5

1. A method for imaging an object with a planar sectioning microscope comprising the steps of:

(a) locating a plane of best focus of the microscope at a first plane within the object;

(b) taking an image of the object at the 10 first plane;

(c) translating the plane of best focus to a second plane within the object;

(d) taking an image of the object at the second plane;

15 (e) removing a slice from the object with a microtome, the slice including a section corresponding to at least one of the first or second planes;

(f) translating the plane of best focus to a third plane within the object; and

20 (g) taking an image of the object at the third plane.

2. The method of claim 1, further comprising the step of, prior to step (a),

25 (h) preparing the object by staining the object with a dye and infiltrating the object with a transparent medium.

3. The method of claim 2, further comprising the step of (i) embedding the object in a transparent medium.

30 4. The method of claim 1, further comprising the step of (j) processing the images detected.

5. The method of claim 1, wherein the planar sectioning microscope is a laser scanning confocal microscope.

35 6. The method of claim 1, further comprising the step of (k) using the images stored to create a model of the object or a plane within the object on a computer screen.

7. An apparatus for optically and mechanically sectioning an object, comprising:

a microtome having a holder for receiving the object, for cutting a slice from the object upon command of a control program;

a planar sectioning microscope mechanically coupled to the microtome by a bench and having a plane of best focus and a narrow depth of focus for imaging the object;

an optical mount mechanically coupled to the planar sectioning microscope and the bench for adjustably translating the plane of best focus relative to the object.

8. The apparatus of claim 7, wherein the planar sectioning microscope is a confocal microscope.

9. The apparatus of claim 8, wherein the confocal microscope is a laser scanning confocal microscope.

10. The apparatus of claim 7, further comprising a film mount for holding film on which images from the planar sectioning microscope may be recorded.

11. The apparatus of claim 9, wherein the laser scanning confocal microscope includes a photodiode detector or a photomultiplier detector.

12. A method for imaging an object, comprising the steps of:

(a) preparing the object;  
(b) placing the object in a holder for viewing by a planar sectioning microscope having a plane of best focus and a narrow depth of focus;

(c) receiving by the planar sectioning microscope an image of a first plane of the object, the first plane located at a plane of best focus;

(d) moving the plane of best focus to a second plane of the object;

(e) receiving via the planar sectioning microscope an image of the second plane of the object;

(f) cutting a slice from the object with a

microtome, the slice including at least one of the first or second planes;

(g) moving the plane of best focus to a third plane of the object;

5 (h) receiving via the planar sectioning microscope an image of the third plane of the object; and

(i) storing the images so received in a computer.

13. The method of claim 12, wherein step (a) 10 includes staining the object with a dye.

14. The method of claim 12, wherein the holder is made of an optically clear material.

15. A method for imaging an object with a planar sectioning microscope, comprising the steps of:

15 (a) receiving by the planar sectioning microscope an image of surface plane of the object;

(b) translating the plane of best focus to a second plane within the object;

20 (c) receiving by the planar sectioning microscope an image of the object at the second plane;

(d) removing a slice from the object with a microtome;

(e) translating the plane of best focus to a third plane of the object; and

25 (f) receiving by the planar sectioning microscope an image of the object at the third plane.

16. A method for imaging an object with a planar sectioning microscope, comprising the steps of:

30 (a) receiving by the planar sectioning microscope an image of a first plane of the object;

(b) translating the plane of best focus by a first increment to a second plane of the object;

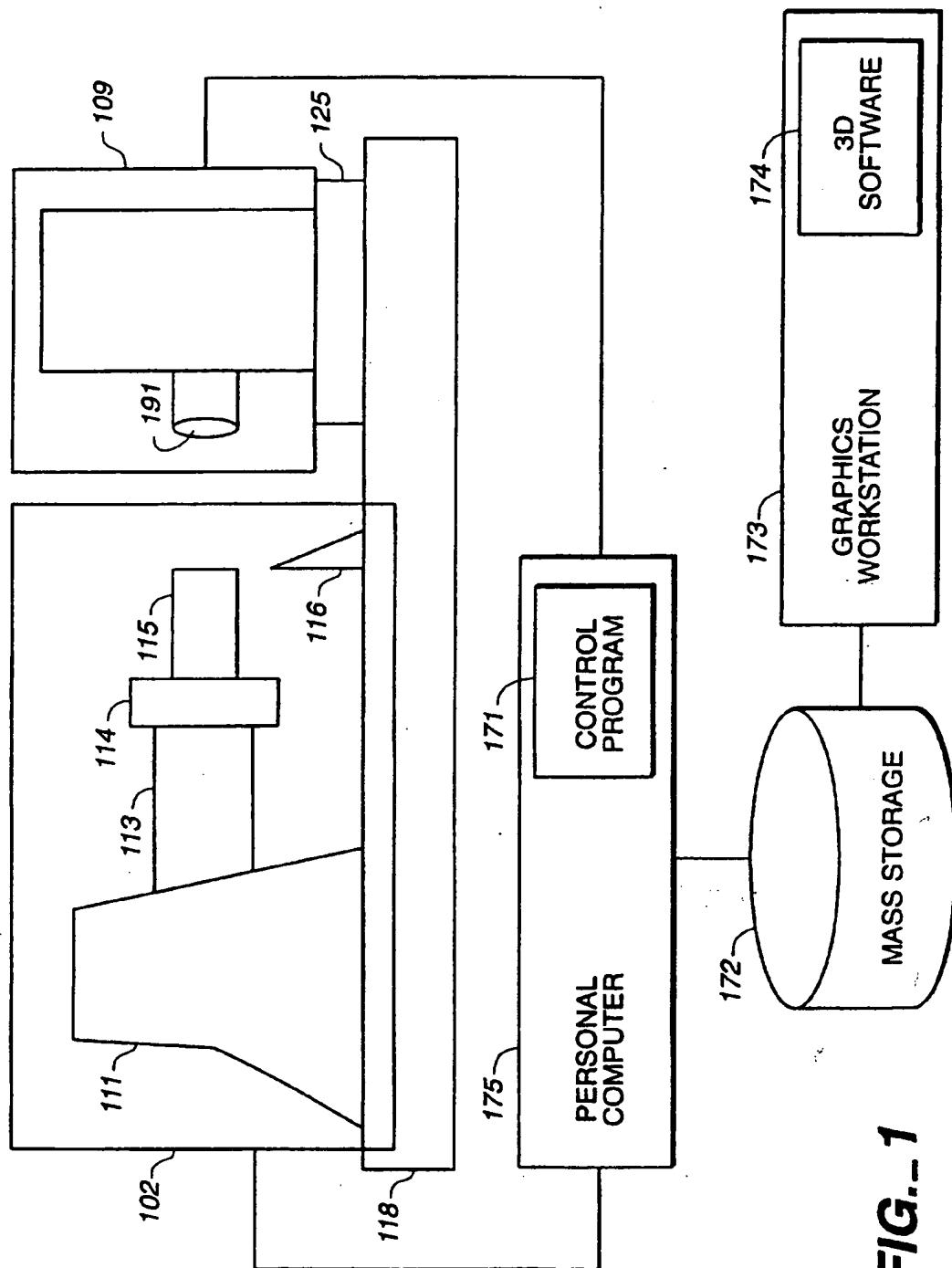
(c) receiving by the planar sectioning microscope an image of the object at the second plane;

35 (d) translating the plane of best focus by a second increment to a third plane of the object;

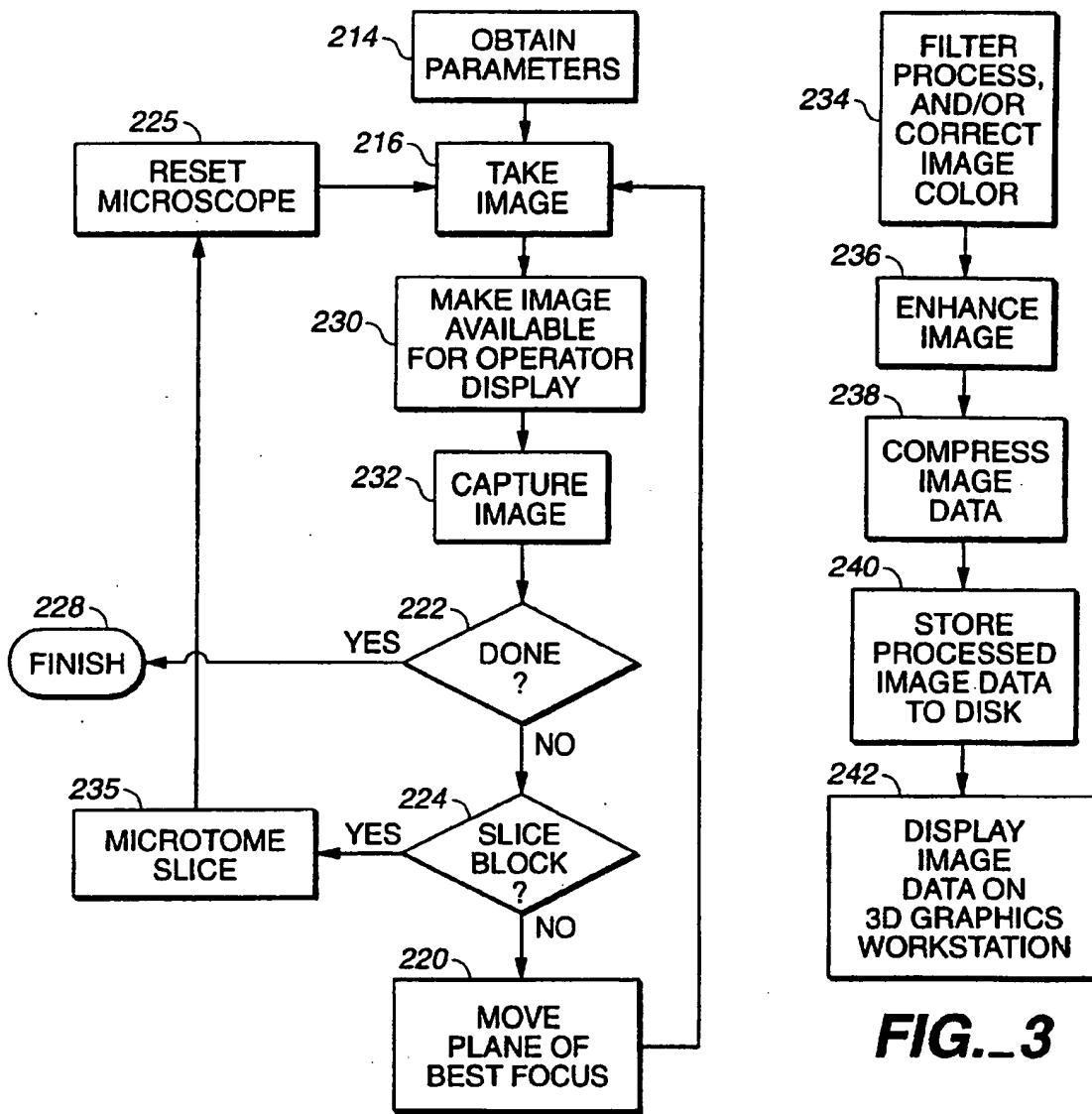
(e) receiving by the planar sectioning microscope an image of the object at the third plane;

- (f) removing a slice from the object with a microtome, the slice including a section corresponding to at least one of the first, second, or third planes;
- 5 (g) translating the plane of best focus to a fourth plane within the object; and
- (h) receiving by the planar sectioning microscope an image of the object at the fourth plane.

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**FIG. 1**

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**FIG.\_2****FIG.\_3**

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/12082

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G06T 17/00

US CL :382/128

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 382/128, 133, 154; 250/201.3; 359/368, 383; 348/379; 83/915.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN

search terms: confocal, sectioning, microscope, microtome

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,960,330 A (KERSCHMANN) 02 October 1990, abstract	1-16
Y	OLDMIXON et al. Overcoming Confocal Scanning Laser Microscopy Depth Limitations. FASEB Journal. 1994. Vol.8:A691. No.5. see entire reference	1-16
Y	ODE et al. A New Technique for Optical 3D Measurements with a Confocal Scanning Laser Microscope. Proceedings of IMTC/94. May 1994. Vol.2. pp.672-676. see entire reference	1-16

 Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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*'P' document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

15 SEPTEMBER 1997

Date of mailing of the international search report

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